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Poliovirus Induces Apoptosis in the Human U937 Promonocytic Cell Line

José Antonio López-Guerrero,[1] María Alonso, Fernando Martín-Belmonte, and Luis Carrasco

Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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The human promonocytic U937 cell line, which is moderately susceptible to poliovirus infection, has been used to investigate the induction of apoptosis by this virus. Infection of U937 cells with poliovirus induces morphological changes typical of apoptosis. Poliovirus-resistant U937 cells (PRU) have been isolated that are resistant to apoptosis induced by poliovirus, but that undergo apoptosis after treatment with TNF plus cycloheximide. Despite the fact that poliovirus triggers nitric oxide production in U937 cells, the inhibitor of inducible nitric oxide (NO) synthase, N^{ω}-monomethyl-L-arginine, did not hinder apoptosis after infection, suggesting that NO does not play a direct role in this process. Finally, poliovirus infection of U937 cells led to the cleavage of pro-caspase-3 and poly(ADP-ribose)polymerase, indicating the activation of the CPP32 ICE-like cysteine protease in the induction of apoptosis. Our findings suggest that cellular death takes place in U937 cells productively infected by poliovirus as a result of apoptosis and involves caspase activation.

Key Words: poliovirus; apoptosis; U937; cell death; PARP; nitric oxide.

INTRODUCTION

Poliovirus infections usually cause the death and lysis of susceptible cells, facilitating the dissemination of viral progeny. After infection of the human gut, poliovirus may reach the central nervous system (CNS), probably involving persistent infection of myeloid cells (Eberle *et al.,* 1995; Freistadt and Eberle, 1996; Freistadt *et al.,* 1993), although this point remains unclear. Despite the fact that morphological changes, known as cytopathic effects (CPE), have been described after infection of cell lines of diverse origins (Bienz *et al.,* 1983; Dales *et al.,* 1965; Haller and Semler, 1995; Schlegel and Kirkegaard, 1995), the nature of the cytopathogenesis of poliovirus on monocytic cells remains poorly understood.

The role of macrophages and macrophage-like cells in the infection of the CNS by some picornaviruses has been investigated in mice (Rossi *et al.,* 1997). Macrophages persistently infected with Theiler's virus may play a critical role in demyelinating disease. Persistent infections of human erythroblastoid (Lloyd and Bovee, 1993) or neuroblastoma cells (Colbère-Garapin *et al.,* 1989) by poliovirus have also been analyzed. In fact, new data suggest that the postpolio syndrome (PPS), in which new muscular symptoms develop decades after acute poliomyelitis, may depend on the persistence of the virus in motoneurons: anti-polio IgM antibodies or poliovirus RNA has been detected in cord spinal fluid (Destombes *et al.,* 1997; Julien *et al.,* 1999).

The interaction between poliovirus and monocyte-like cells has been analyzed using the human promonocytic cell line U937 (López-Guerrero *et al.,* 1989a,b, 1990, 1991a,b; López-Guerrero and Carrasco, 1998). Cell death is greatly delayed in this system, while progeny viruses were made during several days after virus adsorption. The production of poliovirus particles from U937 cells was less than that from other cell lines, such as human fibroblasts. Notably no typical CPE appear in monocytic cells (López-Guerrero *et al.,* 1989a,b, 1991a). Moreover, the infection of U937 by poliovirus produced detectable levels of nitric oxide (NO) without previous activation of the cells (López-Guerrero and Carrasco, 1998). Induction of NO by immunocompetent cells seems to play a role in the modulation of apoptosis, promoting either its induction or its inhibition (Chlichlia *et al.,* 1998; Zhao *et al.,* 1998).

The induction of apoptosis by poliovirus in HeLa cells has been analyzed by Tolskaya *et al.* (1995). Depending on the conditions of infection, poliovirus might induce or prevent apoptosis. Furthermore, two types of cell death have been described after poliovirus infection: canonical cytopathic effects, under productive infections, or caspase-dependent apoptosis, under restrictive conditions (Agol *et al.,* 1998). Thus, the pathways leading to CPE or to apoptosis may be different. Although recent work indicates that poliovirus could induce apoptosis in nervous cells (Girard *et al.,* 1999), it is still unknown whether this virus is able to induce apoptosis in monocytic cells. Taking into account that infection of U937 cells by poliovirus induces both the production of NO and unusual cell death, it was of interest to analyze the activation of apoptosis in this virus–cell system. In this study, we present evidence of apoptosis induction after

[1] To whom correspondence and reprint requests should be addressed. Fax: 34-91-397 4799. E-mail: jalopez@cbm.uam.es.

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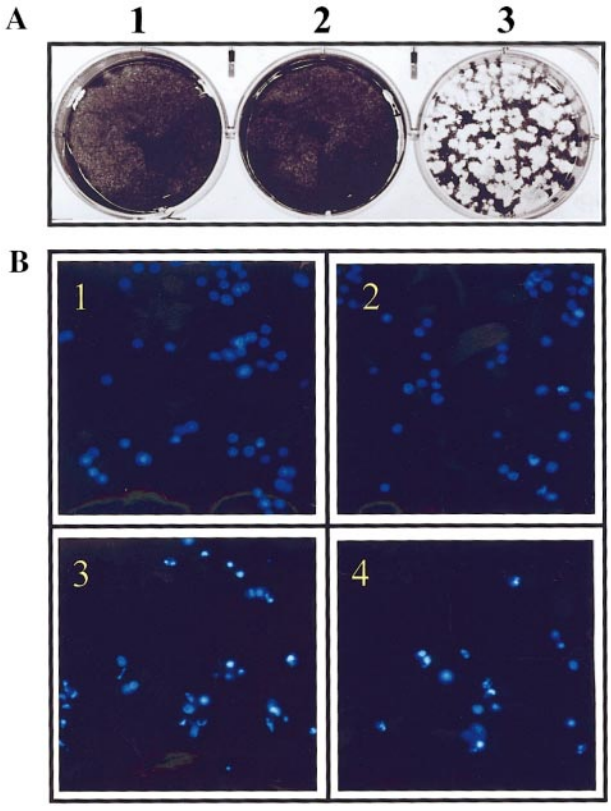

FIG. 1. Nuclear fluorescence analysis of poliovirus-infected cells. U937 cells (1, 3, and 4) or PRU cells (2) were mock-infected (1), infected with poliovirus at 10 PFU/cell (2 and 3), or treated with TNF-α (50 ng/ml) and cycloheximide (10 μg/ml) for 2 h (4). (A) Infectious viral particles were analyzed by standard plaque-assay method at 36 h p.i. (B) Fluorescence microscopy analysis of cells stained with Hoechst solution at 24 h p.i.

poliovirus infection of U937 cells through a mechanism involving activation of caspase-3. Moreover, apoptosis seems to be the only active mechanism of monocyte killing by poliovirus. This represents a good model system for studying the molecular mechanisms of virus-induced apoptosis.

RESULTS

Poliovirus induces apoptosis in U937 cells

The replication of poliovirus in monocytic cells is delayed compared with more permissive cell lines, like HeLa cells (López-Guerrero *et al.,* 1989b,b; López-Guerrero and Carrasco, 1998), such that infectious poliovirus particles appear 36 h postinfection (p.i.) of U937 cells (Fig. 1A). The replication of poliovirus in HeLa cells under restrictive conditions is accompanied by morphological changes characteristic of apoptosis (Agol *et al.,* 1998; Tolskaya *et al.,* 1995). This prompted us to study the mechanisms underlying poliovirus-induced cell death in the human monocytic cell line U937.

U937 cells infected at 10 plaque-forming units (PFU) per cell with poliovirus, enough to infect over 95% of cells (López-Guerrero *et al.,* 1989b), exhibited features typical of apoptosis, such as apoptotic bodies and chromatin condensation (Fig. 1B, panel 3), comparable to those obtained after 2 h of treatment with 50 ng/ml TNF-α and 10 μg/ml cycloheximide (Fig. 1B, panel 4), two known inducers of apoptosis (Rayet *et al.,* 1998). The appearance of apoptotic features correlated with the time course of viral replication previously described (López-Guerrero *et al.,* 1989b). To determine whether or not apoptosis requires active viral infection, poliovirus-resistant U937 cultures (PRU) were isolated after several cycles of infection at a multiplicity of infection (m.o.i.) of 100 PFU per cell and incubation of survivor cells for 3 weeks. As shown in Fig. 1, no infectious virus was detected by plaque assay and no apoptotic bodies were revealed by fluorescence microscopy in PRU cells (Fig. 1B, panel 2). Nevertheless, at an m.o.i. of 10 PFU per cell, the internalization of 50 μg/ml α-sarcin, an indicator of viral entry (Fernández-Puentes and Carrasco, 1980), was similar to that obtained in parental U937 cells, suggesting that PRU cells support viral adsorption. Furthermore, accumulation of viral RNA and proteins was detected at slight amounts at 36 h p.i. (data not shown).

To analyze the ultrastructural modifications of U937 cells in more detail, poliovirus-infected cells were examined by transmission electron microscopy (Fig. 2). While control and poliovirus-infected PRU cells showed normal morphology (Fig. 2, panels 1 and 2), U937 cells exhibited clear signs of apoptosis at 24 h p.i., including extensive chromatin condensation and appearance of apoptotic bodies (Fig. 2, panel 3), similar to those observed after apoptosis provoked by TNF-α and cycloheximide (Fig. 2, panel 4).

Genome fragmentation and caspase activation in poliovirus-infected U937 cells

To test further the occurrence of apoptosis after poliovirus infection of U937 cells, internucleosomal DNA fragmentation was analyzed at 36 h p.i. Figure 3 shows the characteristic nucleosomal ladder comparable to that obtained in TNF-α- and cycloheximide-treated cells. Finally, the characteristic peak that appears by flow cytometry analysis located above the G1 position corresponding to apoptotic cells is also observed in poliovirus-infected U937 cells (Fig. 3A). These results add additional support to the apoptotic fate of poliovirus-infected U937 cells. By contrast, infected PRU cells exhibited no DNA fragmentation at all (data not shown).

Previous findings from our laboratory showed that poliovirus induces NO production in U937 cells (López-Guerrero and Carrasco, 1998). Several reports correlate the accumulation of this gas with apoptosis induction (Bonfoco *et al.,* 1995; López-Guerrero *et al.,* 1997). Thus,

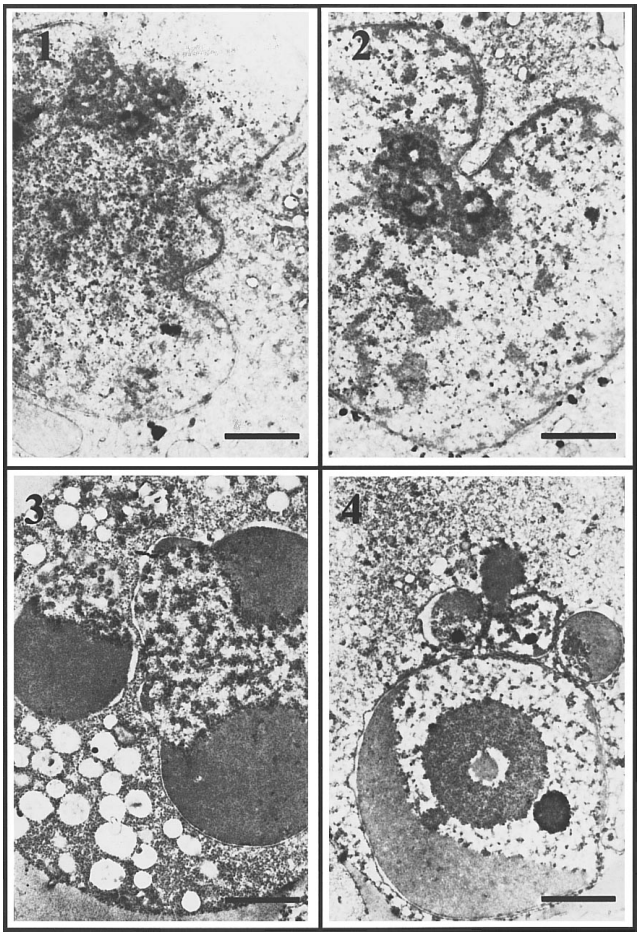

FIG. 2. Electron microscopy analysis of poliovirus-infected cells. U937 cells (1, 3, and 4) or PRU cells (2) were mock-infected (1), infected with 10 PFU per cell of poliovirus over 24 h (2 and 3), or treated with TNF-α (50 ng/ml) and cycloheximide (10 μg/ml) for 2 h (4). Cells were collected by low-speed centrifugation and processed for electron microscopic analysis as described under Materials and Methods. Bars, 1 μm.

we investigated whether induction of NO by poliovirus was related to triggering programmed cell death in U937 cells. As illustrated in Fig. 3B, incubation with a 2 mM concentration of the NO synthase inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA), which blocks the production of NO after viral infection (López-Guerrero *et al.,* 1997; López-Guerrero and Carrasco, 1998), did not modify internucleosomal DNA fragmentation in U937 cells infected with poliovirus at 1 or 10 PFU per cell, suggesting that induction of iNOS does not play a major role in the activation of apoptosis in poliovirus-infected U937 cells.

Poly(ADP-ribose)polymerase (PARP), an enzyme that catalyzes the poly(ADP)ribosylation of nuclear proteins when activated by DNA strand breaks, is cleaved by members of the ICE-like cysteine protease family, in particular by caspase-3, during the onset of apoptosis (Kaufmann *et al.,* 1993; Lazebnik *et al.,* 1994). Since U937 cells express high levels of PARP, cleavage of this protein was studied in poliovirus-infected cells. To this end, U937 or PRU cell extracts were obtained at different times postinfection and analyzed by Western blotting using mouse anti-PARP monoclonal antibody. Figure 4A shows that cleavage of PARP is observed at 12 h p.i., reaching its maximum approximately 12 h later. As a

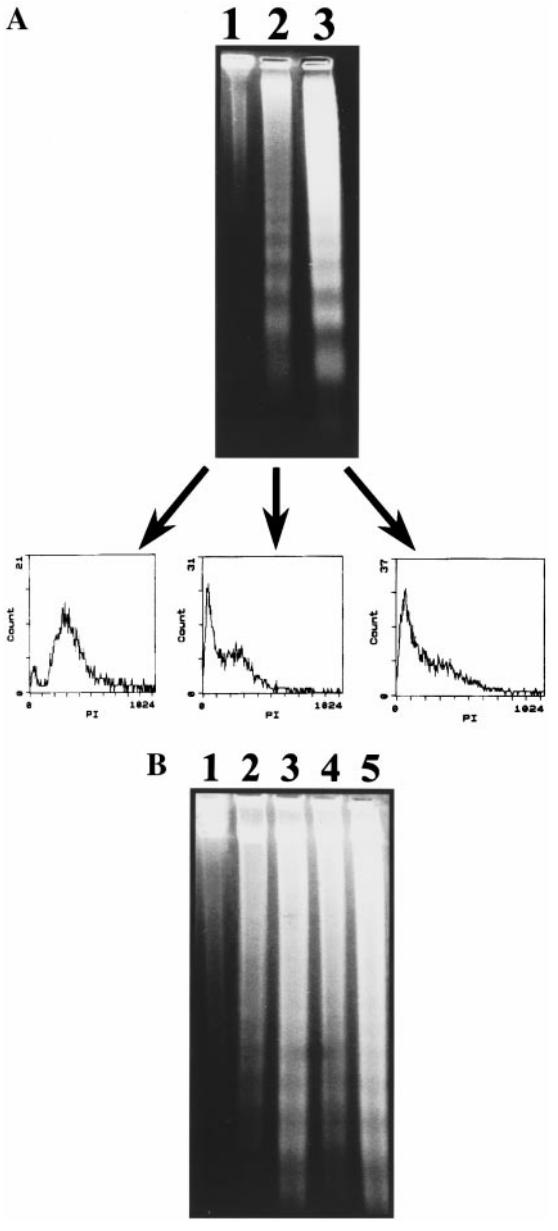

FIG. 3. DNA fragmentation of poliovirus-infected U937 cells. Effect of iNOS inhibition. (A) One million U937 cells were mock-infected (1), infected with poliovirus at 10 PFU/cell over 24 h (2), or treated with TNF-α and cycloheximide for 2 h (3). Cells were collected by low-speed centrifugation and processed for DNA analysis as described under Materials and Methods. Cell distribution is shown in the different stages of the cell cycle, stained with propidium iodide, and determined by flow cytometric analysis. (B) Cells were mock-infected (1) or infected with poliovirus at 1 (2 and 4) or 10 PFU/cell (3 and 5) and pretreated for 4 h with 2 mM L-NMMA (4 and 5) and subsequent DNA analysis was carried out as described for A.

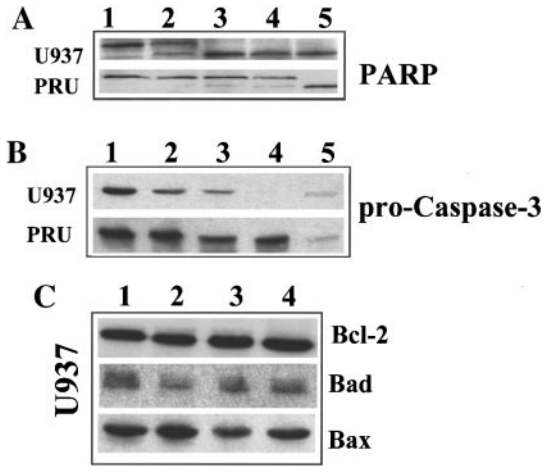

FIG. 4. Cleavage of PARP and pro-caspase-3. Detection of several Bcl-2-family members in poliovirus-infected cells. Total protein extracts were prepared at 0 (1), 12 (2), 24 (3), or 36 h (4) after infection of U937 or PRU cells with poliovirus (10 PFU per cell). Aliquots of 90 μg of protein were analyzed by Western blotting with various antibodies. (A) Monoclonal anti-PARP antibody. The bands corresponding to full-size (116 kDa) or its cleavage product (85 kDa) are indicated. A control of total cleavage of PARP by TNF-α and cycloheximide is indicated (5). (B) Polyclonal anti-caspase-3. The band corresponds to full-length (32 kDa) proenzyme. (C) Monoclonal anti-Bcl-2 and polyclonal anti-Bax and anti-Bad antibodies.

control, total PARP cleavage was obtained with TNF-α and cycloheximide treatment. No cleavage of PARP was detected in poliovirus-infected PRU or mock-infected U937 cells (Fig. 4A). Furthermore, the activation of pro-caspase-3, as a result of its cleavage into two subunits, was analyzed by Western blotting (Fig. 4B). The proenzyme caspase-3 decreases in poliovirus-infected U937 cells or TNF-α- and cycloheximide-treated U937 or PRU cells, correlating with PARP cleavage. Again, no cleavage of pro-caspase-3 was observed in poliovirus-infected PRU cells.

The Bcl-2 protooncogene is considered to be a prototypic regulator of mammalian cell death that may be modulated in U937 cells after apoptosis induction (Brockhaus and Brüne, 1998; Marshall *et al.,* 1999). To test whether poliovirus-induced apoptosis is facilitated by Bcl-2 down-regulation, the levels of this protein were analyzed by Western blotting. Figure 4C shows that there was no variation in the level of Bcl-2 in U937 or PRU cells at different times postinfection. Moreover, analysis of Bax and Bad, two proapoptotic molecules of the Bcl-2 family able to interact with Bcl-2 (Schlesinger *et al.,* 1997), did not exhibit any variation after poliovirus infection (Fig. 4C). Similar results were obtained in PRU cells (data not shown). Taken together, these results suggest that poliovirus-induced apoptosis in U937 cells represents a major Bcl-2-independent mechanism of cell death involving caspase activation.

DISCUSSION

Under unrestricted conditions, infection of permissive cells by poliovirus usually leads to rapid cell death and lysis and development of characteristic pathological changes, defined as cytopathic effects (Bienz *et al.,* 1983; Dales *et al.,* 1965; Haller and Semler, 1995). However, under certain restrictive conditions, poliovirus induces apoptosis (Agol *et al.,* 1998; Tolskaya *et al.,* 1995; Barco *et al.,* 2000). Agol *et al.* (1998) suggested that these two types of cell death by poliovirus are independent of each other. Nevertheless, the situation may be more complex. Thus, other authors have reported that permissive infection with poliovirus may also lead to apoptosis in HeLa cells (Castelli *et al.,* 1997), suggesting that apoptosis was triggered by the 2-5A system through activation of RNase L. In addition, the RNase L-induced apoptosis could be modulated by Bcl-2 (Díaz-Guerra *et al.,* 1997).

We now report that the human monocytic-like cell line U937 undergoes apoptosis after poliovirus infection under permissive conditions. The time course of infection is delayed in this system and viral progeny is less abundant in comparison with other permissive cell lines. Previous results showed that UV-inactivated poliovirus did not affect the viability of U937 cells (López-Guerrero, 1990), suggesting that the induction of apoptosis by poliovirus involves postadsorption mechanisms. Apoptosis seems to be the only mechanism of cell death after poliovirus infection of U937 cells. Moreover, preliminary findings with human primary macrophage cultures infected with poliovirus point to a similar result (data not shown). Cellular variants resistant to poliovirus infection (PRU cells) that are refractory to the induction of apoptosis after infection have been selected. Taking into account that no individual clones were isolated, these results indicate that apoptosis and viral replication run in parallel in these cells.

Consistent with the results of Agol *et al.* (1998), the cleavage of PARP and the proenzyme caspase-3 in poliovirus-infected U937 cells indicates caspase-dependent apoptosis. However, whether this caspase activity is induced by a particular poliovirus product in U937 cells requires further investigation.

Induction of apoptosis in U937 cells after poliovirus infection seems to be Bcl-2-independent, since the degree of accumulation of this oncoprotein remained unchanged, as well as independent of Bax and Bad, two potential partners of Bcl-2. Furthermore, the unmodified high level of Bcl-2 is consistent with the finding that the poliovirus-induced apoptosis is NO-independent, despite the production of detectable amounts of this gas in U937 cells promoted by poliovirus (López-Guerrero and Carrasco, 1998). With regard to this possibility, it is worth noting that the induction of apoptosis in U937 by the NO-releasing compound *S*-nitrosoglutathione could downregulate Bcl-2 (Brockhaus and Brüne, 1998). On the

other hand, and consistent with the present report, Dinsart's group isolated constitutively activated U937 cells, resistant to infection with the parvovirus H-1, which permanently produced large amounts of NO (López-Guerrero *et al.,* 1997). Curiously, these variants were refractory to the apoptosis induction seen in H-1-permissive U937 cells, which did not produce detectable levels of NO after infection (Rayet *et al.,* 1998).

The physiological significance of apoptosis induction by poliovirus in monocytic cells requires further research. After primary infection in the gut, there is evidence that poliovirus interacts with specialized cells that overlie the Peyer's patches (Sicinski *et al.,* 1990; Blondel *et al.,* 1998). Moreover, resident mononuclear phagocytic cells might be the site of the initial rounds of poliovirus replication, since fractionation experiments showed that only monocytes supported poliovirus replication, among PBM cells (Eberle *et al.,* 1995; Freistadt and Eberle, 1996; Freistadt *et al.,* 1993). From the lymphoid tissue of the gut, the virus passes to the bloodstream, causing persistent viremia, which is required for viral spread to the CNS. It is conceivable that under these conditions poliovirus-induced apoptosis represents the most efficient mechanism by which the virus can reach the CNS without activating immune inflammatory responses. Such a mechanism has been advanced for another picornavirus: Theiler's murine encephalomyelitis virus strains BeAn and DGVII (Jelachich *et al.,* 1999; Takata *et al.,* 1998; Tsunoda *et al.,* 1997). This virus causes demyelination by allowing viral persistence in macrophages. Furthermore, recent works suggest that poliovirus may persistently infect murine cells of neuronal origin (Blondel *et al.,* 1998; Destombes *et al.,* 1997) and induce apoptosis (Girard *et al.,* 1999). One of the described mechanisms by which nonretroviral and positive-strand RNA viruses may establish persistence is the abnormal regulation of minus-strand production (Klingel *et al.,* 1992). Surprisingly, poliovirus-infected U937 cells exhibited an abnormal positive:negative viral RNA ratio (López-Guerrero *et al.,* 1991b).

In conclusion, poliovirus induces caspase-dependent apoptosis of the monocytic cell line U937, which seems to be Bcl-2- and NO-independent. This finding may be relevant to the further clarification of viral pathogenesis and to explain virus spread to the CNS.

MATERIALS AND METHODS

Cells and virus

Cells were cultured in RPMI 1640 (Life Technologies, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (FCS) in a 5% CO_2 atmosphere at 37°C. Poliovirus type 1 (Mahoney strain) was propagated in HeLa cells. Virus titration by plaque-assay was performed on HeLa cell monolayers with a final concentration of 0.7% agar (Gibco BRL, Life Technologies). The m.o.i. was expressed as the number of PFU per cell. Experiments were carried out with exponentially growing cultures.

Nucleus Hoechst staining

Cells were allowed to settle for 15 min on slides pretreated with 1 mg of poly-L-lysine (Sigma) per milliliter before fixation with 4% formalin. After being washed with PBS, the slides were incubated with Hoechst stain (Sigma) at a concentration of 75 μg/ml for 30 min at 4°C, washed twice with PBS, and examined at 450 nm after the samples had been excited at 330 nm.

Electron microscopy

Cultures were infected with 10 PFU per cell. Electron microscopy was performed at 24 h p.i. as described previously (Andrés *et al.,* 1997). For conventional Epon embedding, samples were first fixed for 60 min in 2% glutaraldehyde and 2% tannic acid in cacodylate buffer (pH 7.4) and then postfixed with 1% aqueous osmium tetroxide for 30 min. All incubations were performed at room temperature. Specimens were examined at 60 kV and a magnification of ×8000.

DNA fragmentation analysis

Detection of internucleosomal DNA fragmentation was measured by electrophoresis in an agarose gel by meticulously following the method described by Eastman (1995). Briefly, a 2% agarose gel in TBE buffer was prepared. Once the gel solidified, the section immediately above the comb was removed. Subsequently, this section was filled with 1% agarose, 2% SDS, and 64 μg/ml proteinase K in TBE buffer. One million cells were resuspended with 1:1 sample buffer (10% glycerol, 10 mM Tris, pH 8, 0.1% (w/v) bromophenol blue) and RNase A (10 mg/ml) and loaded directly onto the gel. After electrophoresis, the gel was stained with 2 μg/ml ethidium bromide before photographs were taken with Polaroid 665 film. Fluorescence of propidium iodide-stained DNA was quantified with an EPICS-XL flow cytofluorometer (Coulter).

Immunoblot analysis

Cultures (10^6 cells) were infected with poliovirus (10 PFU per cell) or treated for 2 h with 50 ng/ml TNF-α (Sigma) and 10 μg/ml cycloheximide. Cells were collected on several occasions postinfection and suspended in 80-μl samples of buffer (370 mM Tris–HCl, pH 6.8; 17% glycerol, 100 mM DTT, 1% SDS, and 0.024% bromophenol blue). For immunoblot analysis, samples were subjected to SDS–PAGE in 15% acrylamide gels under reducing conditions and transferred to Immobilon-P membranes (Millipore, Bedford, MA). After being blocked with 5% nonfat dry milk, 0.05% Tween 20 in PBS,

blots were incubated with mouse anti-PARP monoclonal antibody (C2-10, PharMingen, San Diego, CA), rabbit anti-caspase-3 polyclonal antibody (PharMingen), mouse anti-Bcl-2 oncoprotein monoclonal antibody (clone 124, Dako, Glostrup, Denmark), rabbit anti-Bax polyclonal antibody (Calbiochem, San Diego, CA), or rabbit anti-Bad polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After several washes, blots were incubated for 1 h with goat anti-mouse IgG antibodies coupled to horseradish peroxidase, washed extensively, and developed using an enhanced chemiluminescence Western blotting kit (ECL, Amersham).

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